

REGULATION ANALYSIS OF ENERGY METABOLISM

MARTIN D. BRAND*

Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, UK

Summary

This paper reviews top-down regulation analysis, a part of metabolic control analysis, and shows how it can be used to analyse steady states, regulation and homeostasis in complex systems such as energy metabolism in mitochondria, cells and tissues. A steady state is maintained by the variables in a system; regulation is the way the steady state is changed by external effectors. We can exploit the properties of the steady state to measure the kinetic responses (elasticities) of reactions to the concentrations of intermediates and effectors. We can reduce the complexity of the system under investigation by grouping reactions into large blocks connected by a small number of explicit intermediates – this is the top-down approach to control analysis. Simple titrations then yield all the values of elasticities and control coefficients within the system. We can use these values to quantify the relative strengths of different internal pathways that act to keep an intermediate or a rate constant in the steady state. We can

also use them to quantify the relative strengths of different primary actions of an external effector and the different internal pathways that transmit its effects through the system, to describe regulation and homeostasis. This top-down regulation analysis has been used to analyse steady states of energy metabolism in mitochondria, cells and tissues, and to analyse regulation of energy metabolism by cadmium, an external effector, in mitochondria. The combination of relatively simple experiments and new theoretical structures for presenting and interpreting the results means that top-down regulation analysis provides a novel and effective way to analyse steady states, regulation and homeostasis in intricate metabolic systems.

Key words: steady state, regulation, homeostasis, regulation analysis, metabolic control analysis, response coefficient, oxidative phosphorylation, energy metabolism, mitochondria, cadmium, hepatocytes, muscle.

Introduction

Metabolic systems (such as a pathway, an organelle, a cell, a tissue or an organism) are often in a steady state, in which the rates of reactions and the concentrations of metabolic intermediates are effectively constant or vary only within narrowly defined limits. For example, the respiration rate of a liver cell under specified conditions does not vary wildly, but remains constant within the normal limits of detection. This steady state is determined by the parameters of the system, such as the nature, kinetics and activities of the enzymes, the temperature and the fixed concentrations of external effector molecules. For a given set of parameters, there is normally a unique steady state. The steady state is maintained by the variables in the system, such as the concentrations of internal metabolites and the rates of the internal reactions. Metabolites directly affect the rates of local enzymes that consume and produce them as substrates and products, and indirectly affect the rates of more distant enzymes by longer-range interactions such as allosteric or covalent effects transmitted through feedback loops. If the steady state wanders, it is the variables that respond and bring it back to its original value.

Regulation of such a metabolic system implies changes in the steady state in response to an external stimulus or stress. For example, the rate of ATP production in skeletal muscle

will increase in response to an appropriate nerve impulse that causes muscle contraction, and the rate of gluconeogenesis in liver will increase in response to raised blood glucagon concentrations. Regulation is mediated by changes in the parameters of the system; for example, a regulatory molecule might change the V_{\max} or K_m of one or more of the enzymes and so alter the unique steady state of the system. A regulatory change is propagated through the system by the variables. For example, changes in the concentrations of the substrate and product of the affected enzymes will have secondary effects on the rest of the system, causing all the other variables to shift to (and then maintain) the new steady state. A special case of regulation, called homeostasis, occurs when the concentration of a particular metabolite is kept constant despite changes in the flux passing through it.

In this article I will discuss regulation analysis (Kacser and Burns, 1973, 1979; Heinrich *et al.* 1977; Kholodenko, 1988; Westerhoff, 1989; Sauro, 1990; Hofmeyr and Cornish-Bowden, 1991; Brand *et al.* 1993; Kahn and Westerhoff, 1993; Hofmeyr *et al.* 1993; Kessler and Brand, 1994*b,c*; Brand and Kessler, 1995), a subset of metabolic control analysis (Kacser and Burns, 1973, 1979; Heinrich and Rapoport, 1974; Fell, 1992; Kacser *et al.* 1995). Regulation analysis has the potential

*e-mail: mdbl@mole.bio.cam.ac.uk.

to provide a quantitative description and deep understanding of how steady states are maintained by the different internal interactions in a complex metabolic system and how regulation by external effectors is propagated from the primary sites of action through different parts of the system to cause it to move to a new steady state.

The steady state

To start with, consider the properties of the steady state in a very simple system: two reactions that convert a fixed concentration of external metabolite X to a fixed concentration of external metabolite Y , through a common intermediate, metabolite M (Fig. 1). If we leave this system alone for long enough (typically, a few seconds or minutes), it will evolve to a steady state where the rate of production of M by the supply reaction is equal to the rate of M consumption by the demand reaction. In this steady state, the system will have a steady rate of conversion of X to Y and a steady concentration of M . If we plot the rates of the two reactions as a function of the concentration of M (Fig. 1), the nature of the steady state becomes clear. The M -consumer will tend to go faster as the concentration of M , its substrate, rises. The M -producer will tend to go slower as the concentration of M , its product, rises. The exact form of the curves is not important for our analysis, and it makes no difference in principle if the kinetics of the enzymes are quite different from the ones shown in Fig. 1. The steady state will occur when the concentration of M evolves to the value where the rate of its production is exactly equal to the rate of its consumption; the intersection of the lines in

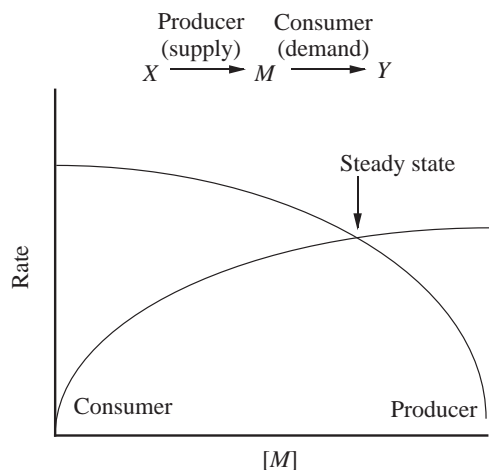


Fig. 1. The steady state. The simple system consists of two reactions (the M -producer and the M -consumer) that share a common internal metabolite M that can vary in concentration. The system is provided with fixed concentrations of external substrate X and external product Y . The graph shows arbitrary relationships between the rates of M production and M consumption as a function of the concentration of M . Where the lines intersect, there is a unique stable steady state with the rate of M production equal to the rate of its consumption. This steady state can only be varied by altering the parameters of the system.

Fig. 1. What happens if the concentration of M is momentarily raised slightly? To the right of the steady state in Fig. 1, demand for M exceeds supply, so the concentration of M drops and the system moves back towards the steady state. The reverse occurs if the concentration of M is lowered slightly: to the left of the steady state in Fig. 1, supply of M exceeds demand and the concentration of M rises towards its steady-state value. In this way, the variables in the system (the concentration of M and the rates of M production and consumption) adjust to maintain the steady state that is dictated by the parameters (the kinetics of the two enzymes, the fixed concentrations of X and Y and the prevailing conditions of pH, temperature and all other external effectors).

We can exploit the steady state to measure the overall kinetic responses to the concentration of M of each of the two enzymes. We can make a parameter change that alters the relationship between the rate of the M -producer and the concentration of M ; for example, we could raise or lower the concentration of the enzyme, or treat it with an activator or with a specific inhibitor that lowers its V_{\max} or decreases its affinity for M . If we inhibit, the system will evolve to a new steady state with a decreased concentration of M and decreased rates of both the M -consumers and the M -producers (Fig. 2). The new steady state will not lie on the line describing the original kinetic response of the M -producer to M , because we have interfered with the kinetics of this reaction. However, it will lie on the line describing the original kinetic response of the M -consumer to M , because we have not altered the kinetics of this reaction, but only changed the concentration of its substrate. By progressively inhibiting the M -producer and measuring the rate of the system and the concentration of M , we can observe successive steady states that map out the kinetic response of the M -consumer to M as shown in Fig. 2. This strategy gives us a simple way to measure the kinetic

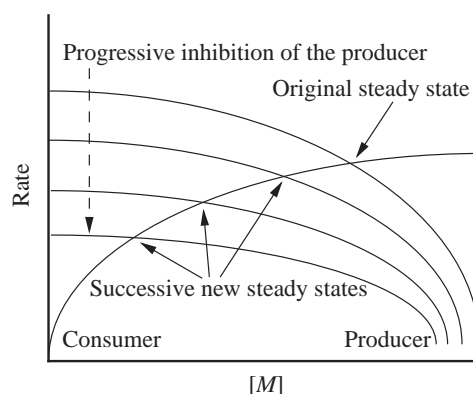


Fig. 2. Measurement of the kinetic response of the M -consumer to the concentration of M . The kinetic parameters of the M -producer are changed (in this example, by adding an inhibitor specific for the producer reaction). The system evolves to a new steady state that lies at a different point on the original line describing the kinetic response of the M -consumer to M . By progressively inhibiting the producer through successive steady states, a series of values of rate and $[M]$ are obtained that fully describe the kinetics of the M -consumer in the range investigated.

response of the consumer to the concentration of M , *in situ*, whatever form the kinetic response may take. We can also use this strategy to measure the kinetic response of the producer: in a separate series of experiments, we can successively alter the kinetics of the M -consumer and map out the kinetic response of the M -producer to M as shown in Fig. 3. An alternative strategy is to add a new reaction that consumes or produces M and independently to measure the steady-state rates of the two original reactions as a function of the concentration of M . Either strategy gives a full kinetic description of the steady state from simple measurements of rates and concentrations.

This description of the steady state and the experimental approach to measure the kinetics of the enzymes involved is essentially simple and straightforward. However, the simplicity is deceptive because the description provides the basis for a very powerful theoretical and experimental analysis of the regulation of complex biological systems, as described below.

Complex pathways

So far, I have considered a very simple pathway with two enzymes and a single intermediate. What about more complex, real pathways? Because we empirically measure the overall kinetic response of a reaction to M with all other effectors kept constant, the reactions can have any type of kinetics without affecting the analysis. So we can group several reactions together into a larger block of reactions and label them the M -producers or the M -consumers and carry out the analysis as if they were a single (complicated) enzyme. Grouping reactions together in this way is the basis of top-down metabolic control analysis, which simplifies complex pathways and makes them more amenable to experimental application of control analysis (Kacser and Burns, 1973; Heinrich and Rapoport, 1974; Kacser, 1983; Westerhoff *et al.* 1984, 1987; Bohnensack, 1985; Fell and Sauro, 1985; Westerhoff and van Dam, 1987; Brand *et al.* 1988; Brown *et al.* 1990a; Schuster *et al.* 1993; Brand and Brown, 1994; Brand, 1996). In fact, a block of reactions such as the M -producers can be of any complexity, containing enzymes, transporters, non-enzymic steps, many intermediates, compartments, feedback loops and allosteric interactions. There are some rules to be observed. First, there must be no intermediates within one block of reactions that have any direct effects on another block; any such intermediates must be considered explicitly (Ainscow and Brand, 1995). Second, when inhibitors are used to titrate steady states, they must have sites of action (of known or unknown type) that are confined to blocks whose kinetics are not being measured in that experiment (Brown *et al.* 1990a). The degree of complexity that is allowed to remain after the system is divided into blocks will reflect a balance between the need for an experimentally simple system and a theoretically interesting one.

The complex networks that make up metabolism are dramatically illustrated by formalised maps of dots to represent

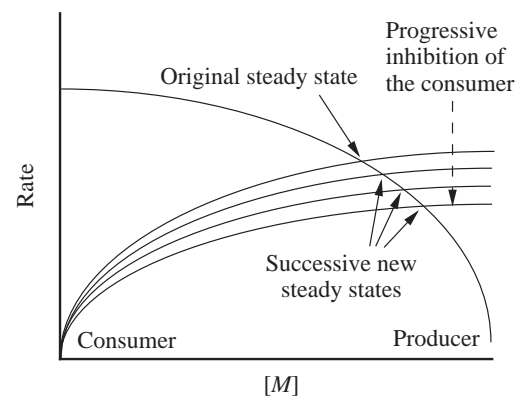


Fig. 3. Measurement of the kinetic response of the M -producer to the concentration of M . The kinetic parameters of the M -consumer are changed (in this example, by adding an inhibitor specific for the consumer reaction). The system evolves to a new steady state that lies at a different point on the original line describing the kinetic response of the M -producer to M . By progressively inhibiting the consumer through successive steady states, a series of values of rate and $[M]$ are obtained that fully describe the kinetics of the M -producer in the range investigated.

metabolites connected by lines to represent reactions catalysed by enzymes (Alberts *et al.* 1994). Such maps have several hundred dots and lines and it is intimidating to try to describe the overall pattern of regulation in such large networks. By grouping the reactions into large blocks connected by only a few metabolites using the top-down approach, the whole problem becomes much more tractable. Fig. 4 shows schemes that have been analysed in this laboratory. They represent some of the many ways to group the reactions of metabolism in mitochondria, cells and tissues so that maintenance of steady states in energy metabolism and their regulation may be quantified.

The simplest system comprises two blocks of reactions connected by a single intermediate, as shown in Fig. 1 and Fig. 4A. To analyse branched systems containing three or more blocks of reactions connected by a single intermediate, as in Fig. 4B–D, it is necessary to split up one of the blocks of Fig. 1 or Fig. 4A and to measure the separate sub-fluxes independently, but otherwise the analysis is essentially unchanged. Thus, for Fig. 4B, the kinetics of the M -producers can be found by measuring the steady states as described above during titration of either of the M -consumers with inhibitors, and the kinetics of each of the M -consumers can be found by measuring their individual rates in the steady states achieved during inhibitory titration of the M -producers. Alternatively, the kinetics of all three blocks can be measured by inserting a branch and measuring the dependence of the rates of the three original blocks on the concentration of M as $[M]$ is varied by varying the new branch. Schemes with more than one serial metabolic intermediate, as in Fig. 4E, can be solved by combining the results of different groupings of the system, in this case by combining results of the analysis of Fig. 4A–C. Schemes with parallel intermediates (Fig. 4F–H) can be solved by carrying out double titrations and then analysing the data to

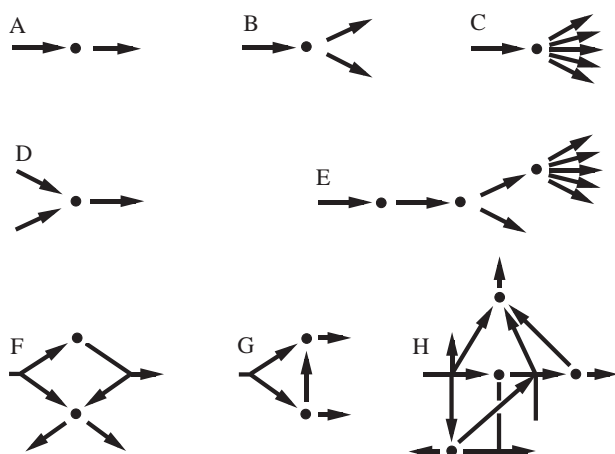


Fig. 4. Some different ways to group the reactions of energy metabolism. Dots represent explicit intermediates and lines represent blocks of reactions that interact with them. (A) A representation of the simplest case, for example state 4 respiration in mitochondria, cells or tissues, where the intermediate is protonmotive force produced by substrate oxidation and consumed by proton leak (Brand *et al.* 1988; Brown *et al.* 1990b; Buttgereit *et al.* 1994; Rolfe and Brand, 1996), or β -oxidation, where the intermediate is the acetyl CoA/CoA ratio (Quant *et al.* 1993). (B) Active respiration where protonmotive force is consumed by both proton leak and the phosphorylation reactions (Hafner *et al.* 1990b; Brown *et al.* 1990b; Buttgereit *et al.* 1994; Rolfe and Brand, 1996). (C) ATP production and its consumption by several blocks of ATP-consumers (Buttgereit and Brand, 1995). (D) Reduction of ubiquinone by different dehydrogenases and its reoxidation by the electron transport chain (C. Buckley and M. D. Brand, unpublished observations). (E) Energy flow through NADH, protonmotive force and ATP in hepatocytes (Brown *et al.* 1990b; S. J. Price and M. D. Brand, unpublished observations). (F) Flow through cytochrome *c* and protonmotive force in isolated mitochondria (Ainscow and Brand, 1995). (G,H) More complex flows in hepatocytes, with ATP, protonmotive force, NADH and pyruvate as explicit intermediates (E. K. Ainscow and M. D. Brand, unpublished observations).

show (i) the dependence of the rate of each block of reactions on the concentration of the first intermediate at a fixed concentration of the second intermediate and (ii) the dependence of the rate of each block of reactions on the concentration of the second intermediate at a fixed concentration of the first. Other, more subtle, approaches can also be used to achieve the same result without having to make the full set of measurements.

So, very complex pathways can be dealt with by grouping reactions together and solving the simple pathways that result. As the questions that are posed by the investigator become more complex, then the systems to be analysed will tend to become more complex too, but the simple versions and the simple questions are best tackled first. This approach is quite different from the more traditional 'bottom-up' approach, in which we start from a particular effect, such as the observed inhibition of an enzyme by a metabolite *in vitro*, and try to discover whether this effect is important in regulation in a cell or an organism.

Elasticities and control coefficients

As discussed above, we can readily analyse the kinetic interactions taking place in the steady state in an intricate metabolic web that we have conceptually simplified by grouping it into a few large blocks of reactions interconnected through a few intermediates. How do we make quantitative statements about control of steady states and about regulation?

First, we simplify the kinetics. The only parts of the kinetic curves of Fig. 1 that actually apply in the steady state are the slopes at the steady-state intersection point, so we can use these slopes instead of taking the whole curves. For simple Michaelis–Menten enzymes, this simplification is not a great advantage, but for enzymes with more complicated kinetics, and for groups of enzymes, it enables us to continue the analysis without getting bogged down in mechanistic and kinetic details that are not necessary to the understanding of overall system behaviour under the condition of interest. The slopes of the kinetic plots, when normalised and expressed as fractional changes in rate caused by fractional changes in metabolite concentration with all other effectors kept constant, are known as elasticities, symbolised ϵ . Elasticities change as the concentration of M changes and apply only under the conditions specified, but they accurately describe the kinetics of the enzymes or blocks of reactions in the steady state.

Control coefficients, symbolised C , quantify the amount of control that a single reaction or a block of reactions has over a rate or over a metabolite concentration in the system. A flux control coefficient expresses the fractional change in flux that would be caused by an infinitesimal fractional change in the activity of the block; a concentration control coefficient expresses the fractional change in a metabolite concentration that would be caused in the same way. Small values denote little control, whereas values near 1 show that the block has strong control. The strength of control exerted by a particular block of reactions depends on the kinetics of all the blocks in the system; in other words, the flux control coefficients are functions of (and can be calculated from) all the elasticities and rates.

The way that control depends on the elasticities should be clear from Fig. 5, which examines the distribution of flux control in the simple two-block system in a steady state where the elasticities of the two blocks differ. Consider what happens if we make a change in the activity of the M -producers so their instantaneous rate drops from the steady-state rate to point a . The rate of production of M is now less than the rate of its consumption, so the system evolves to a new steady state at b , with a lower concentration of M . The new steady state lies at the intersection of the line describing the original kinetics of the M -consumers with the (dashed) line ab describing the new kinetics of the M -producers. Because the elasticity (slope) of the M -consumers to M is low compared with the elasticity of the M -producers, there has to be a fairly large decrease in the concentration of M to cause the M -consumers to change rate and this decrease in $[M]$ relieves some of the inhibition on the remaining activity of M -producers, so opposing the original change quite effectively. The final steady-state rate after a

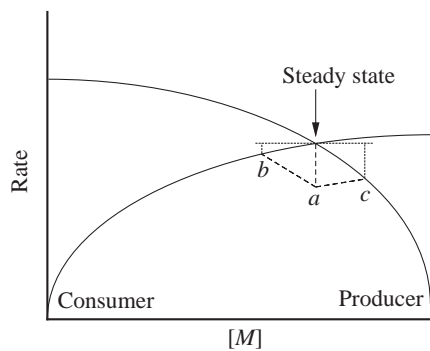


Fig. 5. The relationship between elasticities and control coefficients. At the steady state, the consumer reactions do not change rate very much when $[M]$ changes; this block has a low elasticity to M . A decrease in the activity of the consumers to bring their local rate to a is followed by an evolution of the steady state to c , so there is a relatively large rate change in the whole system and the consumer block has a large flux control coefficient. Conversely, the producers have a high elasticity to M . A decrease in the activity of the producers to bring their local rate to a is followed by an evolution of the steady state to b , so there is a relatively minor rate change and the producers have a small flux control coefficient.

change in the M -producers (at b) is not very different from the original one. In other words, the M -producers have a fairly low flux control coefficient. Now consider the effect of an equivalent change in the activity of the M -consumers so that their instantaneous rate drops from the steady-state rate to point a . The rate of production of M now exceeds its rate of consumption, so the system evolves to a new steady state at c with a higher concentration of M . The new steady state lies at the intersection of the line describing the original kinetics of the M -producers with the (dashed) line ac describing the new kinetics of the M -consumers. Because the elasticity (slope) of the M -consumers to M is low compared with the elasticity of the M -producers, the increase in the concentration of M is sufficient to cause a large rate change in the M -producers and the original change in the consumers is opposed rather ineffectively. The final steady-state rate after a change in the M -consumers (at c) is very different from the original one: the M -consumers have a fairly high flux control coefficient. So blocks with low elasticities towards the intermediate have high flux control coefficients, and *vice versa*. It turns out that the ratio of the elasticities is the inverse of the ratio of the flux control coefficients. In general, we can quantify the control exerted by each block of reactions over all the fluxes and concentrations in a complex pathway from simple measurements of elasticities and rates in the way described above.

Elasticity analysis

The method discussed above provides a simple experimental way to discover the primary site or sites of action of an effector that interacts with the system. By comparing the kinetic curves for the different reaction blocks in the presence and absence of

the effector, it is a simple matter to identify which blocks have a changed kinetic response to the intermediate (and so are primary targets of the effector) and which do not (and so change rate only as a secondary consequence of the transmission of signals within the system by changes in the system variables). For example, Fig. 2 demonstrates that the inhibitor that was used acts only on the producer reactions and not on the consumers, because the producers have altered kinetics but the consumers do not. This approach is known as top-down elasticity analysis (Hafner *et al.* 1990a; Brand, 1990, 1993; Brand *et al.* 1993). If more than one block of reactions is a primary target (as in Fig. 6), the relative sizes of the different primary effects can be seen at a glance from the kinetic plots (Harper and Brand, 1993, 1995; Harper *et al.* 1993).

How do real effectors work on metabolic systems? In most cases, there will be single sites of action, or only a few sites in a complicated network, so that the actions of the effector are transmitted through the system by changes in the concentrations of metabolites, and homeostasis is not achieved. For full homeostasis, the effector would need to change the rates in the system without perturbing the concentrations of any of the metabolites. This desirable end can be achieved by making the same absolute change simultaneously in the activities of all the enzymes in a pathway. For a simple two-block pathway, Fig. 6 shows that, if the M -producers and the M -consumers are both reduced in activity by the same amount, the steady-state rate can be altered without any change in the concentration of M , so that homeostasis of M is achieved. The same argument applies to more complicated networks; for every additional metabolite whose concentration is homeostatically maintained, there must be at least one extra change in the activity of an enzyme. Similarly, appropriate changes in the activities of the blocks could give the same rate at a different concentration of the intermediate (Fig. 6). This homeostatic approach may be the ideal way to change the rate of production of an end product (or the concentration of an intermediate) without affecting the rest of the metabolism of a cell, and is the basis of Kacser's 'Universal Method' for biotechnological engineering (Kacser and Acerenza, 1993; Small and Kacser, 1994). Evolution may also have favoured the approach of modifying rates but not metabolite concentrations in the central metabolic pathways: this results in control being widely distributed (Fell and Thomas, 1995) and requires that physiological intracellular effectors such as Ca^{2+} or phosphorylation cascades act at several steps simultaneously (Korzeniewski *et al.* 1995). Activation of operons, and the grouping of pathways into organelles such as mitochondria, reflects the same principles.

Regulation analysis

Now that we have a simple way to measure elasticities and control coefficients, we have all the tools needed to conduct a full and quantitative analysis of steady states, regulation and homeostasis in complicated metabolic networks.

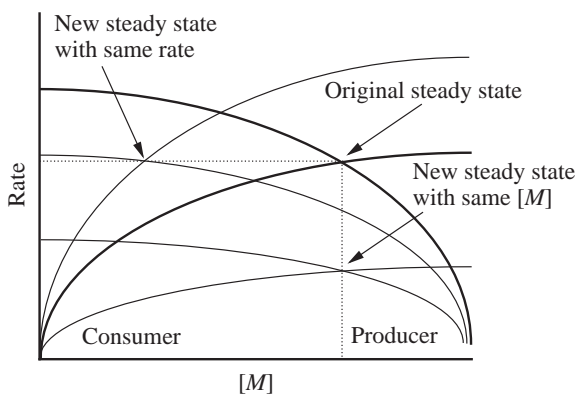


Fig. 6. How steady-state rates can be altered without changing the concentration of the intermediate. If both consumer and producer are altered by the same absolute amount, the new steady state has the original concentration of M but a different rate (homeostasis). Conversely, by making appropriate changes in the activities of the two blocks, it is also possible to maintain the original rate at a very different concentration of M .

Internal control of steady states

To describe the maintenance of steady states, we need to quantify how strongly a particular metabolite or a particular block of reactions within the system controls the rate of a reaction or the concentration of a metabolite. We also want to be able to measure and compare the relative importance of the different routes within the network by which the effects are transmitted. All of these descriptions are available within regulation analysis by direct measurement or by multiplying together the appropriate elasticities and control coefficients (Westerhoff, 1989; Sauro, 1990; Hofmeyr and Cornish-Bowden, 1991; Brand *et al.* 1993; Kahn and Westerhoff, 1993; Hofmeyr *et al.* 1993; Kesseler and Brand, 1994b; Brand and Kesseler, 1995).

We may wish to describe how strongly a system metabolite controls a steady-state flux by its different actions within the network. This is encapsulated in an internal response coefficient. For example, we might want to quantify how ATP concentration controls the steady-state rate of glycolysis through its effects on phosphofructokinase and to compare the importance of this control pathway with the effects exerted through hexokinase and other enzymes (Westerhoff, 1989). The elasticity to ATP quantifies how much a block of reactions responds to ATP, and the flux control coefficient over glycolysis quantifies how much the block controls glycolytic rate, so if we multiply the two together we can calculate the partial internal response coefficient, or regulatory strength (Kacser and Burns, 1973; Kahn and Westerhoff, 1993), which describes how strongly ATP controls glycolytic rate through its effects on that block. By comparing the regulatory strengths of different blocks, we can say which actions of ATP are most important for the control of glycolytic rate, which actions are significant but less important and which actions are insignificant. The negative effects of ATP at some blocks will exactly cancel the positive effects at other blocks because we

are looking at a steady state where there is no net tendency for ATP to change the system to a new state, so the sum of the partial internal response coefficients, symbolised R , will always be zero.

We can use the same approach to determine how strongly a metabolite controls the concentration of another metabolite through its interactions with different blocks of reactions. By multiplying the elasticity of a block to the first metabolite by the concentration control coefficient of the block over the second metabolite, we can calculate the partial internal response coefficients through that route. Comparison of these regulatory strengths shows us which routes are important for transmission of control through the system. For example, we could analyse the relative importance of ATP control over glucose 6-phosphate concentration through its different effects on blocks of reactions that produce or consume glucose 6-phosphate.

There is a special case in which we are interested in the routes by which a metabolite controls its own concentration, to ask the questions: which blocks of reactions respond strongly to perturbations in the metabolite's concentration and tend to buffer it, and which blocks respond weakly? For example, does ATP control its own concentration most strongly through its interactions with the reactions that produce it or with the reactions that consume it? This partial internal response coefficient (after a sign change) has been called the homeostatic strength (Kahn and Westerhoff, 1993). It is calculated by multiplying the elasticity of a block to the metabolite by the concentration control coefficient of the block over the metabolite. Partial internal response coefficients of a metabolite over its own concentration sum to -1 .

Alternatively, we may be interested in the way that a block of reactions controls the flux through another block. For example, how does glycolysis control the rate of oxidative phosphorylation in the steady state? It does so through various intermediates, so if we multiply the concentration control coefficient of the first block over an intermediate by the elasticity of the second block to that intermediate, we will calculate a measure of how strongly the first block controls the flux through the second one by this route. By comparing the effects through different metabolites, we can accurately describe which intermediates in the system are most important in the control of the second block by the first. This type of partial internal response coefficient (after normalisation to produce a sum of 1) has been called a partitioned regulatory coefficient (Sauro, 1990). The positive and negative effects will sum to the control coefficient of the first block over the flux through the second block.

Regulation

To describe regulation and homeostasis, we need to quantify how strongly an effector that is external to the system controls a rate or the concentration of a metabolite within the system. As before, we also want to be able to measure and compare the strengths of the different routes within the network by which the effects are transmitted. Once again, all of these descriptions are available within regulation analysis by direct

measurement or by multiplying together the appropriate elasticities and control coefficients (Kacser and Burns, 1973; Kholodenko, 1988; Hofmeyr and Cornish-Bowden, 1991; Kahn and Westerhoff, 1993; Hofmeyr *et al.* 1993; Kessler and Brand, 1994c; Brand and Kessler, 1995).

An external effector will change system rates and concentrations. Its effects are characterised by a set of control coefficients, the response coefficients, that describe the fractional change in a system variable caused by an infinitesimal fractional change in the effector. Each block of reactions that is a primary target of an external effector will have an elasticity to the effector; the other blocks will have zero elasticity to it. The elasticity quantifies the effect on that block, so if we multiply it by the control coefficient of the block over a system rate or a system metabolite concentration, we can calculate how much the effector changes the rate or concentration through its effects on the block. This is the partial response coefficient. The partial response coefficients will sum to the overall response coefficient of the system to the external effector, and their relative values illuminate the importance of each route through the system in causing the overall response. If desired, the way that the effects of a regulatory molecule on a block or on a metabolite are transmitted through the rest of the system can be quantified for small effects using the partial internal response coefficients described above.

If there is homeostasis of a metabolite, the partial response coefficients of its concentration to the effector will sum to zero even though the flux through the metabolite has changed. The values of the partial response coefficients will quantitatively describe the different routes through which homeostasis is achieved.

Regulation analysis of energy metabolism

Regulation analysis can give a complete description of steady states, regulation and homeostasis in complex metabolic networks and requires only elasticities and control coefficients as its raw data. To what systems has it been applied? Hofmeyr and Cornish-Bowden (1991) and Kahn and Westerhoff (1993) give worked examples based on hypothetical reaction schemes, but the practical application of the approach has so far been restricted to the study of energy metabolism in mitochondria, cells and tissues. More widespread use should begin when the power and simplicity of the method become more widely appreciated. The fullest experimental regulation analysis that has been published quantifies the control of different steady states of oxidative phosphorylation in isolated potato tuber mitochondria and their regulation by cadmium, a toxic pollutant with multiple sites of action (Kessler and Brand, 1994a,b,c; Brand and Kessler, 1995).

Internal control of steady states of energy metabolism

Kessler and Brand (1994a) grouped energy metabolism in potato tuber mitochondria into three blocks of reactions (substrate oxidation, the proton leak pathway through the membrane and the phosphorylation reactions) connected by a shared intermediate, protonmotive force. Protonmotive force is a

thermodynamic quantity describing the electrochemical potential for protons across the mitochondrial inner membrane, but it can be treated just like a more conventional metabolite within control analysis (Westerhoff and van Dam, 1987). Kessler and Brand (1994b) measured the partial internal response coefficients of the three system rates to the value of protonmotive force over a range of states from resting, with no ATP synthesis (state 4), to active, with maximum rates of ATP synthesis (state 3), at different concentrations of cadmium. The analysis showed how the effects of protonmotive force propagate through the system by activatory and inhibitory routes, some strong and some weak, to control the steady state. For example, protonmotive force regulates respiration rate very strongly in state 4 through its antagonistic stimulatory effect on the proton leak and its inhibitory effect on substrate oxidation, with partial internal response coefficients (regulatory strengths) of 8.1 and -8.1 respectively (Table 1). However, protonmotive force regulates respiration rate much more weakly in state 3, through its stimulatory effect on phosphorylation and its antagonistic inhibitory effect on substrate oxidation, with almost no effect through proton leak (partial internal response coefficients of 1.3, -1.3 and 0.0 (actually 0.001) respectively (Table 1). To give an illustrative analogy, we could say that in state 4 internal regulation of respiration rate is taut, with strong opposing forces, but in state 3 it is slack, with rather weak opposing forces. With cadmium present, internal regulation in state 4 was much weaker and comparable to internal regulation in state 3.

Kessler and Brand (1994b) also analysed the internal regulation of protonmotive force. They showed how the protonmotive force controls its own value strongly through its interaction with substrate oxidation in state 4, with control through the interaction with the proton leak also having a significant role (Table 1). As oxidative phosphorylation is increased to state 3, the pattern of internal regulation shifts so that, in state 3, protonmotive force controls its own value mostly through its interactions with the phosphorylation reactions and only 20% through substrate oxidation, interactions with proton leak having an insignificant role. At high cadmium concentrations, however, the interactions through the proton leak remain important in all states.

From published elasticities and control coefficients, it is possible to calculate the partial internal response coefficients that describe internal regulation in a number of experimental systems of energy metabolism in mitochondria, cells and tissues. Table 1 shows such recalculated values for systems grouped into one supply block and one demand block or into one supply block and two demand blocks. The values listed under the heading ${}^iR_M^J$ are the partial internal response coefficients over the supply block exerted by the common intermediate *via* block *i*. In two-block systems, protonmotive force (or membrane potential, which is its major component) has strong regulatory strength over respiration rate in rat liver mitochondria and in hepatocytes, but rather weaker regulatory strength in thymocytes and intact skeletal muscle. The measured intermediates in fatty acid metabolism have very much weaker control over the β -oxidation pathway. In the

Table 1. *Partial internal response coefficients*

System	Intermediate	${}^1R_M^I$	${}^2R_M^I$	${}^3R_M^I$	${}^1R_M^M$	${}^2R_M^M$	${}^3R_M^M$	Reference
A Systems with one supply block ($i = 1$) and one demand block ($i = 2$)								
RLM, state 4	Δp	-5.2	5.2	-	-0.66	-0.34	-	Brand <i>et al.</i> (1988)
PTM, state 4	Δp	-8.1	8.1	-	-0.55	-0.45	-	Kessler and Brand (1994b)
Hepatocytes, state 4								
Euthyroid	$\Delta\psi$	3.3	3.3	-	-0.83	-0.17	-	Harper and Brand (1993)
Hypothyroid	$\Delta\psi$	-6.5	6.3	-	-0.90	-0.10	-	Harper and Brand (1993)
Hyperthyroid	$\Delta\psi$	2.0	2.0	-	-1.11	-0.14	-	Harper and Brand (1994)
Thymocytes, state 4	$\Delta\psi$	1.5	1.5	-	-0.95	-0.05	-	Buttgereit <i>et al.</i> (1994)
Skeletal muscle, state 4	$\Delta\psi$	1.0	0.8	-	-0.61	-0.39	-	Rolfe and Brand (1996)
RLM, uncoupled	AcCoA/CoA	-0.1	0.1	-	-0.28	-0.72	-	Quant <i>et al.</i> (1993)
RLM, uncoupled	acylCoA/CoA	-0.2	0.2	-	-0.63	-0.37	-	Kunz (1991)
RLM, uncoupled	NADH/NAD	-0.1	0.1	-	-0.05	-0.95	-	Kunz (1991)
B Systems with one supply block ($i = 1$) and two demand blocks ($i = 2$) and ($i = 3$)								
PTM, state 3	Δp	-1.3	1.3	0.0	-0.20	-0.80	0.00	Kessler and Brand (1994b)
Hepatocytes, resting								
Fed	$\Delta\psi$	2.2	1.0	1.4	-0.68	-0.14	-0.16	Brown <i>et al.</i> (1990b)
Starved	$\Delta\psi$	1.7	0.6	1.0	-0.73	-0.12	-0.15	Brown <i>et al.</i> (1990b)
Euthyroid	$\Delta\psi$	3.9	0.6	3.2	-0.53	-0.06	-0.37	Harper and Brand (1993)
Hypothyroid	$\Delta\psi$	5.8	1.6	4.4	-0.67	-0.06	-0.17	Harper and Brand (1993)
Hyperthyroid	$\Delta\psi$	3.0	0.5	2.5	-0.59	-0.06	-0.35	Harper and Brand (1994)
Thymocytes, ConA	$\Delta\psi$	1.9	0.6	1.3	-0.88	-0.05	-0.13	Buttgereit <i>et al.</i> (1994)
Skeletal muscle, resting	$\Delta\psi$	0.8	0.5	0.3	-0.74	-0.32	-0.19	Rolfe and Brand (1996)

In most cases, partial response coefficients were calculated as the product of the appropriate elasticities and control coefficients given in the cited references. Partial response coefficients over fluxes should sum to 0, partial response coefficients over the concentration of M should sum to -1 ; where they do not, it is due to rounding errors in the calculation of the values using the published truncated elasticities.

${}^iR_M^I$, partial response coefficient of flux J (this Table contains values for control over flux 1 only) to metabolite M operating through block i ; ${}^iR_M^M$, partial response coefficient of metabolite M to its own concentration operating through block i ; RLM, rat liver mitochondria; state 4, state with no oxidative phosphorylation of ADP to ATP; PTM, potato tuber mitochondria; state 3, state with maximal oxidative phosphorylation of ADP to ATP; ConA, Concanavalin A; Δp , protonmotive force; $\Delta\psi$, membrane potential; AcCoA, acetyl Coenzyme A; acyl CoA, acyl Coenzyme A.

three-block systems of energy metabolism in cells and tissues, respiration rate is controlled by membrane potential through inhibitory effects on substrate oxidation, balanced by antagonistic stimulatory effects exerted through both demand blocks, with the phosphorylation reactions tending to be more prominent. Analyses of this type should be even more illuminating when experimental values of elasticities and control coefficients for more complex systems with several intermediates become available.

Regulation of energy metabolism

Kessler and Brand (1994c) have analysed the regulation of oxidative phosphorylation in isolated potato tuber mitochondria by cadmium. The overall response coefficients to cadmium of substrate oxidation, proton leak, phosphorylation, protonmotive force and effective P/O ratio show complex effects; for example, cadmium activates respiration rate under some conditions but inhibits it under others. By measuring the elasticities of the three blocks of reactions in the system to cadmium, and multiplying them by the control exerted over system variables by each block, partial response coefficients were calculated. These partial response

coefficients give a full picture of the regulation of energy metabolism by cadmium in this experimental system. For example, they quantify how the respiration rate is regulated by cadmium through its direct inhibitory effect on substrate oxidation and its direct stimulatory effect on the proton leak. Under conditions in which the leak has much of the control over respiration rate, such as state 4 and high cadmium concentrations, the stimulatory effect is quantitatively more important, and there is an increase in respiration rate. Under other conditions in which the elasticity of substrate oxidation to cadmium is higher and the control by the proton leak is lower, the inhibitory effect dominates and there is a decrease in respiration rate. The regulation analysis allows the regulatory loops within this simple system to be unravelled and understood in their system context, providing a powerful insight into how the primary effects on the system are relayed by the secondary effects to give the observed behaviour.

Conclusions

Regulation analysis is the application of metabolic control analysis that allows one to give quantitative answers to the

traditional questions of regulation in biochemistry and physiology. How does a system achieve stability in the steady state? How is it regulated by external effectors such as hormones that shift it to a new steady state? How does it achieve homeostasis of some internal metabolite concentrations in the face of changes in flux caused by external effectors? If elasticities and control coefficients can be measured, regulation analysis allows the quantitative description of each of these conditions. Maintenance of steady states is described by partial internal response coefficients that describe the strength of the various interactions of enzymes and intermediates within the system that keep it in a steady state. Regulation is described by partial (external) response coefficients that describe in detail how the system changes in response to its external environment. Regulatory influences can be followed through a complex network of metabolic reactions to see which routes contribute most strongly to a particular response and which are not so important. When regulation operates homeostatically to maintain the concentration of an intermediate, the strengths of the different routes that lead to homeostasis can be described, measured and compared.

Elasticities and control coefficients can be calculated from the kinetic information in the literature, so if we could be confident that all the relevant kinetic interactions have been correctly measured *in vitro*, then a mathematical model of the system can be constructed that allows a full regulation analysis to be performed to explain the system behaviour completely. Such mathematical models have value, but generally we cannot be totally confident of the input parameters, so the models need to be rigorously checked and refined by comparing their descriptions with the real world. The alternative approach discussed here is to simplify the system by grouping reactions together, then to analyse the simpler system experimentally. Information about the fine detail of the regulation will be lost, but it can be added back later if it is needed. If top-down regulation analysis is carried out correctly, the steady-state and regulatory properties of a complex biochemical or physiological system can be accurately quantified relatively easily.

References

- AINSCOW, E. K. AND BRAND, M. D. (1995). Top-down control analysis of systems with more than one common intermediate. *Eur. J. Biochem.* **231**, 579–586.
- ALBERTS, B., BRAY, D., LEWIS, J., RAFF, M., ROBERTS, K. AND WATSON, J. D. (1994). *Molecular Biology of the Cell*, 3rd edn. New York, London: Garland. p. 83.
- BOHNENSACK, R. (1985). Theory of steady-state control in complex metabolic networks. *Biomed. biochim. Acta* **44**, 1567–1578.
- BRAND, M. D. (1990). The proton leak across the mitochondrial inner membrane. *Biochim. biophys. Acta* **1018**, 128–133.
- BRAND, M. D. (1993). Control of oxidative phosphorylation in liver mitochondria and cells: top-down control analysis and top-down elasticity analysis. In *Surviving Hypoxia: Mechanisms of Control and Adaptation* (ed. P. W. Hochachka, P. L. Lutz, T. Sick, M. Rosenthal and G. van den Thillart), pp. 295–309. Boca Raton: CRC Press.
- BRAND, M. D. (1996). Top down metabolic control analysis. *J. theor. Biol.* (in press).
- BRAND, M. D. AND BROWN, G. C. (1994). The experimental application of control analysis to metabolic systems. In *Biothermokinetics* (ed. H. V. Westerhoff), pp. 27–35. Andover: Intercept.
- BRAND, M. D., CHIEN, L.-F. AND ROLFE, D. F. S. (1993). Regulation of oxidative phosphorylation. *Biochem. Soc. Trans.* **21**, 757–762.
- BRAND, M. D., HAFNER, R. P. AND BROWN, G. C. (1988). Control of respiration in non-phosphorylating mitochondria is shared between the proton leak and the respiratory chain. *Biochem. J.* **255**, 535–539.
- BRAND, M. D. AND KESSELER, A. (1995). Control analysis of energy metabolism in mitochondria. *Biochem. Soc. Trans.* **23**, 371–376.
- BROWN, G. C., HAFNER, R. P. AND BRAND, M. D. (1990a). A 'top-down' approach to the determination of control coefficients in metabolic control theory. *Eur. J. Biochem.* **188**, 321–325.
- BROWN, G. C., LAKIN-THOMAS, P. L. AND BRAND, M. D. (1990b). Control of respiration and oxidative phosphorylation in isolated rat liver cells. *Eur. J. Biochem.* **192**, 355–362.
- BUTTGEREIT, F. AND BRAND, M. D. (1995). A hierarchy of ATP-consuming processes in mammalian cells. *Biochem. J.* **312**, 163–167.
- BUTTGEREIT, F., GRANT, A., MÜLLER, M. AND BRAND, M. D. (1994). The effects of methylprednisolone on oxidative phosphorylation in Concanavalin-A-stimulated thymocytes. Top down elasticity analysis and control analysis. *Eur. J. Biochem.* **223**, 513–519.
- FELL, D. (1992). Metabolic control analysis: a survey of its theoretical and experimental development. *Biochem. J.* **286**, 313–330.
- FELL, D. A. AND SAURO, H. M. (1985). Metabolic control and its analysis. Additional relationships between elasticities and control coefficients. *Eur. J. Biochem.* **148**, 555–561.
- FELL, D. A. AND THOMAS, S. (1995). Physiological control of metabolic flux: the requirement for multisite modulation. *Biochem. J.* **311**, 35–39.
- HAFNER, R. P., BROWN, G. C. AND BRAND, M. D. (1990a). Thyroid-hormone control of state-3 respiration in isolated rat liver mitochondria. *Biochem. J.* **265**, 731–734.
- HAFNER, R. P., BROWN, G. C. AND BRAND, M. D. (1990b). Analysis of the control of respiration rate, phosphorylation rate, proton leak rate and protonmotive force in isolated mitochondria using the 'top-down' approach of metabolic control theory. *Eur. J. Biochem.* **188**, 313–319.
- HARPER, M.-E., BALLANTYNE, J. S., LEACH, M. AND BRAND, M. D. (1993). Effects of thyroid hormones on oxidative phosphorylation. *Biochem. Soc. Trans.* **21**, 785–792.
- HARPER, M.-E. AND BRAND, M. D. (1993). The quantitative contributions of mitochondrial proton leak and ATP turnover reactions to the changed respiration rates of hepatocytes from rats of different thyroid status. *J. Biol. Chem.* **268**, 14850–14860.
- HARPER, M.-E. AND BRAND, M. D. (1994). Hyperthyroidism stimulates mitochondrial proton leak and ATP turnover in rat hepatocytes but does not change the overall kinetics of substrate oxidation reactions. *Can. J. Physiol. Pharmacol.* **72**, 899–908.
- HARPER, M.-E. AND BRAND, M. D. (1995). Use of top-down elasticity analysis to identify sites of thyroid hormone-induced thermogenesis. *Proc. Soc. exp. Biol. Med.* **208**, 228–237.
- HEINRICH, R., RAPOPORT, S. M. AND RAPOPORT, T. A. (1977). Metabolic regulation and mathematical models. *Prog. Biophys. molec. Biol.* **32**, 1–82.
- HEINRICH, R. AND RAPOPORT, T. A. (1974). A linear steady-state treatment of enzymatic chains. *Eur. J. Biochem.* **42**, 89–95.

- HOFMEYR, J.-H. S. AND CORNISH-BOWDEN, A. (1991). Quantitative assessment of regulation in metabolic systems. *Eur. J. Biochem.* **200**, 223–236.
- HOFMEYR, J.-H. S., CORNISH-BOWDEN, A. AND ROHWER, J. M. (1993). Taking enzyme kinetics out of control; putting control into regulation. *Eur. J. Biochem.* **212**, 833–837.
- KACSER, H. (1983). The control of enzyme systems *in vivo*: elasticity analysis of the steady state. *Biochem. Soc. Trans.* **11**, 35–40.
- KACSER, H. AND ACERENZA, L. (1993). A universal method for achieving increases in metabolite production. *Eur. J. Biochem.* **216**, 361–367.
- KACSER, H. AND BURNS, J. A. (1973). The control of flux. *Symp. Soc. exp. Biol.* **27**, 65–104.
- KACSER, H. AND BURNS, J. A. (1979). Molecular democracy: who shares the controls? *Biochem. Soc. Trans.* **7**, 1149–1160.
- KACSER, H., BURNS, J. A. AND FELL, D. A. (1995). The control of flux. *Biochem. Soc. Trans.* **23**, 341–366.
- KAHN, D. AND WESTERHOFF, H. V. (1993). The regulatory strength: how to be precise about regulation and homeostasis. *Acta biotheor.* **41**, 85–96.
- KESSELER, A. AND BRAND, M. D. (1994a). Localisation of the sites of action of cadmium on oxidative phosphorylation in potato tuber mitochondria using top-down elasticity analysis. *Eur. J. Biochem.* **225**, 897–906.
- KESSELER, A. AND BRAND, M. D. (1994b). Effects of cadmium on the control and internal regulation of oxidative phosphorylation in potato tuber mitochondria. *Eur. J. Biochem.* **225**, 907–922.
- KESSELER, A. AND BRAND, M. D. (1994c). Quantitative determination of the regulation of oxidative phosphorylation by cadmium in potato tuber mitochondria. *Eur. J. Biochem.* **225**, 923–935.
- KHOLODENKO, B. N. (1988). How do external parameters control fluxes and concentrations of metabolites? An additional relationship in the theory of metabolic control. *FEBS Lett.* **232**, 383–386.
- KORZENIEWSKI, B., HARPER, M.-E. AND BRAND, M. D. (1995). Proportional activation coefficients during stimulation of oxidative phosphorylation by lactate and pyruvate or by vasopressin. *Biochim. biophys. Acta* **1229**, 315–322.
- KUNZ, W. S. (1991). Application of the theory of steady-state flux control to mitochondrial β -oxidation. *Biomed. biochim. Acta* **50**, 1143–1157.
- QUANT, P. A., ROBIN, D., ROBIN, P., GIRARD, J. AND BRAND, M. D. (1993). A top-down control analysis in isolated rat liver mitochondria: can the 3-hydroxy-3-methylglutaryl-CoA pathway be rate-controlling for ketogenesis? *Biochim. biophys. Acta* **1156**, 135–143.
- ROLFE, D. F. S. AND BRAND, M. D. (1996). Proton leak and control of oxidative phosphorylation in perfused, resting rat skeletal muscle. *Biochim. biophys. Acta* **1276**, 45–50.
- SAURO, H. M. (1990). Regulatory responses and control analysis: assessment of the relative importance of internal effectors. In *Control of Metabolic Processes* (ed. A. Cornish-Bowden and M. L. Cardenas), pp. 225–230. New York: Plenum Press.
- SCHUSTER, S., KAHN, D. AND WESTERHOFF, H. V. (1993). Modular analysis of the control of complex metabolic pathways. *Biophys. Chem.* **48**, 1–17.
- SMALL, J. R. AND KACSER, H. (1994). A method for increasing the concentration of a specific internal metabolite in steady-state systems. *Eur. J. Biochem.* **226**, 649–656.
- WESTERHOFF, H. V. (1989). Control, regulation and thermodynamics of free-energy transduction. *Biochimie* **71**, 877–886.
- WESTERHOFF, H. V., GROEN, A. K. AND WANDERS, R. J. A. (1984). Modern theories of metabolic control and their application. *Biosci. Rep.* **4**, 1–22.
- WESTERHOFF, H. V., PLOMP, P. J. A. M., GROEN, A. K., WANDERS, R. J. A., BODE, J. A. AND VAN DAM, K. (1987). On the origin of limited control of mitochondrial respiration by the adenine nucleotide translocator. *Archs Biochem. Biophys.* **257**, 154–169.
- WESTERHOFF, H. V. AND VAN DAM, K. (1987). *Thermodynamics and Control of Biological Free Energy Transduction*. Amsterdam: Elsevier. 568pp.